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16. ABSTRACT

Rat embryos undergoing organogenesis (day 10.5 of pregnancy) were exposed to 1.0 mM saccharin or 0.1, 0.3, or 1.0 mM-cyclohexylamine in an in vitro metabolic activating system, 1.0 mM-saccharin had few adverse effects on yolk-sac growth or embryo growth or differentiation. However, rat embryos cultured in vitro with cyclohexylamine and an adult rat hepatic microsomal activating system showed more growth retardation and dysmorphogenicity than embryos exposed to the same concentrations of cyclohexylamine alone. A concentration of 1.0 mM-cyclohexylamine alone reduced yolk-sac DNA content (by 51%), embryo DNA content (69%) and crown-rump length (26%). In the presence of a hepatic microsomal activating system this same dose of cyclohexylamine decreased yolk-sac DNA (60%), embryo DNA (84%) and crown-rump length (49%). The effects of the metabolic activating system and of cyclohexylamine were additive. Other than decreasing final embryo DNA content, exposure to 0.1 and 0.3 mM-cyclohexylamine did not produce any deleterious effects on in vitro rat embryo growth or differentiation. Thus a substantial concentration of saccharin (1.0 mM) or cyclohexylamine (0.3 mM) in vitro did not elicit markedly deleterious effects on rat organogenesis, a result in keeping with published in vivo findings.

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STUDIES OF SACCHARIN AND CYCLOHEXYLAMINE IN A COUPLED MICROSOMAL ACTIVATING/EMBRYO CULTURE SYSTEM

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Abstract—Rat embryos undergoing organogenesis (day 10.5 of pregnancy) were exposed to 1.0 mM-saccharin or 0.1, 0.3 or 1.0 mM-cyclohexylamine in an *in vitro* metabolic activating/embryo culture system. Either in the absence or presence of a metabolic activating system, 1.0 mM-saccharin had few adverse effects on yolk-sac growth or embryo growth or differentiation. However, rat embryos cultured *in vitro* with cyclohexylamine and an adult rat hepatic microsomal activating system showed more growth retardation and dysmorphogenicity than embryos exposed to the same concentrations of cyclohexylamine alone. A concentration of 1.0 mM-cyclohexylamine alone reduced yolk-sac DNA content (by 51%), embryo DNA content (69%) and crown–rump length (26%). In the presence of a hepatic microsomal activating system this same dose of cyclohexylamine decreased yolk-sac DNA (60%), embryo DNA (84%) and crown–rump length (49%). The effects of the metabolic activating system and of cyclohexylamine were additive. Other than decreasing final embryo DNA content, exposure to 0.1 and 0.3 mM-cyclohexylamine did not produce any deleterious effects on *in vitro* rat embryo growth or differentiation. Thus a substantial concentration of saccharin (1.0 mM) or cyclohexylamine (0.3 mM) *in vitro* did not elicit markedly deleterious effects on rat organogenesis, a result in keeping with published *in vivo* findings.

INTRODUCTION

During the 1970s, the technique of *in vitro* whole-embryo culture was greatly improved and popularized (New, 1978; Steele & New, 1974). The relevance of this method for mechanism studies and the assessment of embryotoxicity and teratogenicity was further enhanced by the successful coupling of an adult rat hepatic metabolism system to embryo culture (Kitchin, Sanyal & Schmid, 1981a). Subsequently, the *in vitro* toxicity and teratogenicity of four water-miscible and two water-immiscible solvents were described (Kitchin & Ebron, 1983). This development makes possible the *in vitro* study of water-insoluble reproductive toxins. Finally, these two advances in embryo-culture techniques were integrated in the *in vitro* study of diallate (Ebron & Kitchin, 1982), a water-insoluble compound which requires metabolic activation to express mutagenic and carcinogenic activity (Sandhu & Waters, 1980; Schuphan, Rosen & Casida, 1979).

The *in vitro* embryotoxicity and teratogenicity data correspond well with existing *in vivo* literature (New, 1978). However, many more positive compounds have been tested *in vitro* than compounds lacking reproductive or teratogenic effects. For that reason, two well-studied negative compounds, saccharin and cyclohexylamine (a cyclamate metabolite) were selected for *in vitro* study. *In vivo* studies of both of these compounds in the rat have demonstrated a lack of embryotoxicity and teratogenicity. In mice, however, two studies have shown cyclohexylamine to be embryotoxic (Becker & Gibson, 1970; Kroes, Peters, Berkvens *et al.* 1977). *In vivo* both saccharin (Matthews, Fields & Fishbein, 1973; Sweatman & Renwick, 1980) and cyclohexylamine (Renwick & Williams, 1972) are excreted rapidly by the kidney. The *in vitro* whole-embryo culture system lacks this pathway of maternal excretion and ability to lower progressively the serum concentration of these agents. It is therefore of interest to see whether these two compounds are more toxic *in vitro* than *in vivo*.

EXPERIMENTAL

Chemicals. Saccharin (*o*-benzoic sulphimide, 98 + %) and cyclohexylamine (97%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Cyclohexylamine was dissolved in sterile distilled water and saccharin was dissolved in 0.1 N-NaOH; both solutions were titrated to pH 7.4 with 0.1 N-HCl.

Embryo culture. Sprague–Dawley CD rats were obtained from Charles River Breeding Laboratories, Kingston, NY. During the afternoon (3.00 to 5.00 pm) of pregnancy day 10.5 (the morning the female is found to be sperm positive is called day 0.5), embryos which had rotated into concave foetal position (Witschi stage 16) were collected from several rats (usually 4–6 animals) and were allocated to one of the various treatment groups. Embryos from the same dam were placed in different treatment groups to avoid litter effects. Embryos were cultured for 48 hr in rotating 30-ml serum bottles according to methods previously described (Kitchin *et al.* 1981a; New, 1978; Steele & New, 1974). Each embryo was singly cultured in 5 ml of incubation medium containing serum, obtained from 75–80-day-old male rats, 50 μg streptomycin/ml and 50 IU penicillin/ml (GIBCO, Grand Island, NY) in an O_2:CO_2:N_2 atmosphere of 20:5:75 (v/v) for 20 hr and of 40:5:55 (v/v) for the next 28 hr (Sanyal & Wiebke, 1979).

Metabolic activating system. Hepatic microsomes from adult Sprague–Dawley CD male rats were obtained as described previously (Kitchin, Schmid & Sanyal, 1981b) and used on the day of preparation. This avoids any loss of metabolic activity that may occur during freezing, storage or thawing (Dent, Schnell, Graichen *et al.* 1981). The microsomal activating system consisted of microsomes (0.35–0.50 mg protein/ml) and 1.0 mM-NADPH and was added at the beginning of the 48-hr culture period.

Treatment groups. Rat embryos (day 10.5) were exposed to saccharin (1.0 mM) or cyclohexylamine (0.1, 0.3 or 1.0 mM) for the entire 48-hr culture period, either with or without a microsomal activating system.

At the end of the 48-hr culture period, embryos were removed from the medium and washed in 5 ml Ringer's solution. The yolk-sac diameter, the rate of the heart beat and the somite number were recorded using a binocular stereomicroscope. On the basis of gross observation, embryos were classified as either normal or abnormal when compared to untreated embryos cultured *in vitro*. The embryonic flexion, heart, neural tube, brain development, optic, otic and olfactory systems, branchial bars, maxillary and mandibular processes were examined for the assessment of morphology. Absence of an embryonic heart beat was used as the criteria of death. If the viability of control embryos fell below 65% on a given experimental day, the experiment was discarded.

The embryo and yolk sac dissected from each conceptus were frozen and stored at −20°C until required (within 2–3 wk) for DNA determinations. These tissues were homogenized in 1 ml 0.05 M-phosphate buffer, pH 7.4, with a sonifier (Branson Sonic Power Co.). DNA was determined by the ethidium bromide fluorescence method after digestion of aliquots of homogenate with RNAase and protease (Boer, 1975).

Statistics. Quantitative data are represented as means ± SEM for each treatment group; significance was evaluated using two-way analysis of variance of the Statistical Analysis System (Snedecor & Cochran, 1967). Enumerative data are expressed as the incidence of occurrence; the statistical significance was calculated using Fischer's exact test (Goldstein, 1964).

RESULTS

The growth and differentiation of rat embryos exposed to 1.0 mM-saccharin, with or without a metabolic activating system, was generally not significantly different from controls (Fig. 1; Table 1). Of twenty parameters tested, a decrease in the size of the yolk-sac diameter ($P < 0.05$) was the only significant finding in embryos cultured in serum containing 1.0 mM-saccharin. The addition of a microsomal activating system did not potentiate the *in vitro* effect of saccharin. In this study the metabolic activating system *per se* led to some deleterious effects in the yolk sac. Significant decreases in yolk-sac diameter ($P < 0.01$) and final yolk-sac DNA content ($P < 0.05$) occurred in the metabolically activated control although embryonic parameters were not significantly affected.

At concentrations of 0.1 and 0.3 mM without metabolic activation the cyclamate metabolite cyclohexylamine did not show any biological effects except for a decrease in embryo DNA content by 41 and 32%, respectively, compared to controls ($P < 0.05$; Fig. 1). At 1.0 mM-cyclohexylamine, eight of nine embryonic growth and development parameters were adversely affected; embryo DNA content decreased by ~69%. Embryonic viability was unaffected. Ten of the twelve rat embryos differentiating in 1.0 mM-cyclohexylamine were abnormal in morphology ($P < 0.01$), while only two of twelve control embryos were scored as abnormal. Thus with cyclohexylamine, abnormal embryo morphogenesis occurred at doses that were not lethal to the embryo during the 48-hr culture period.

In the presence of a microsomal activating system, cyclohexylamine was substantially more toxic. The yolk sacs and embryos exhibited little growth in media containing 1.0 mM-cyclohexylamine, hepatic microsomes and 1.0 mM-NADPH. Normally, tenfold growth occurs in embryo and yolk-sac DNA during the 2-day culture period. In ten of eleven embryos cultured with 1.0 mM-cyclohexylamine, microsomes and NADPH, the morphogenesis was so poor that the somite number could not be counted. Most of the embryos (nine of eleven) were judged to be dead on the basis of a lack of heart beat at the end of culture. In this treatment group all quantitative yolk sac and embryonic variables were significantly less ($P < 0.01$

Table 1. Embryotoxic and dysmorphogenic effects of saccharin and cyclohexylamine *in vitro*

Treatment		Crown–rump length (mm)	Somite number	Total no. of embryos	Live embryos	Abnormal live embryos
Compound	Dose (mM)					
Without metabolic activation						
Control	—	5.55 ± 0.22	35.0 ± 1.29	13	12	2
Saccharin	1.0	5.28 ± 0.32	35.0 ± 0.88	9	8	1
Cyclohexylamine	0.1	5.55 ± 0.23	35.7 ± 0.80	6	5	0
	0.3	5.30 ± 0.22	34.3 ± 0.87	7	7	2
	1.0	4.08 ± 0.19**	28.6 ± 0.92**	13	12	10*
With metabolic activation						
Control	—	5.21 ± 0.20	33.4 ± 0.72	18	14	3
Saccharin	1.0	5.07 ± 0.17	32.7 ± 1.18	9	8	4
Cyclohexylamine	0.1	4.76 ± 0.21	32.3 ± 0.76	14	12	6
	0.3	4.67 ± 0.22	31.2 ± 1.20	12	9	6
	1.0	2.65 ± 0.11*	NM	11	2*	2

NM = Not measureable

Values (totals or means ± SEM for total live embryos) are marked with asterisks where they differ significantly from the control (two-way analysis of variance): *$P < 0.01$; **$P < 0.001$.

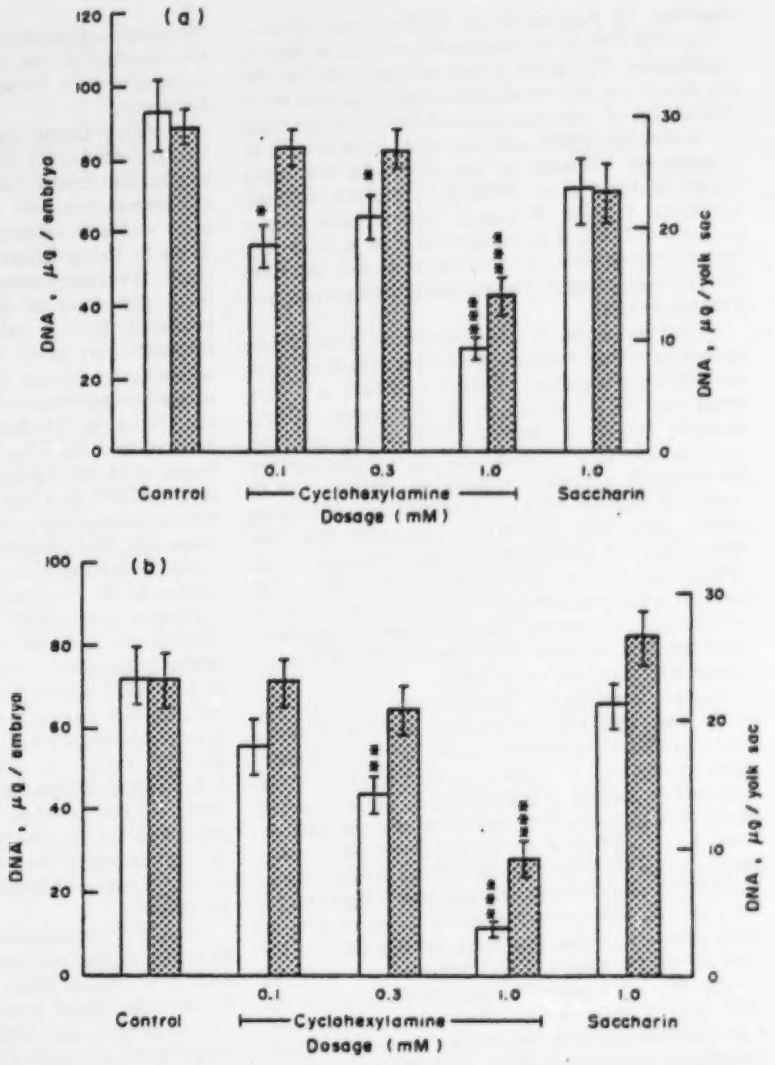

Fig. 1. DNA content of embryos □ and yolk sacs ■ from whole embryos taken from rats on day 10.5 of gestation and exposed *in vitro* for 48 hr to saccharin or cyclohexylamine at the concentrations indicated (a) in the absence and (b) in the presence of male rat hepatic microsomes and 1.0 mM NADPH. Values are means ± SEM for 6–13 samples (see Table 1 for numbers/group) and those marked with asterisks differ significantly from the control (two-way analysis of variance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

or $P < 0.001$) than either the controls, controls with metabolic activation, or the group given 1.0 mM cyclohexylamine alone. Although the difference was not statistically significant, the incidence of abnormal embryos at all three doses of cyclohexylamine was higher in the presence of a microsomal activating system than in cultures exposed to cyclohexylamine alone (Table 1). A two-way analysis of variance showed that the interaction between the metabolic activating system and cyclohexylamine was additive; no evidence for potentiation was seen.

In this study 78% of the abnormal embryonic development was seen in the head region. Most commonly the telencephalon failed to become bulbous and to protrude from the mesencephalon. In the control group, a twisted tail and mis-shapen back were the only abnormalities observed. In the controls cultured with a metabolic activation system, all three abnormal embryos showed poor head development. In the cyclohexylamine groups with a microsomal activating system, head abnormalities were the most frequently observed developmental effect, but swollen mandibles and a mis-shapen back were seen in two embryos.

DISCUSSION

In the present study, yolk-sac DNA content was significantly depressed in the presence of a hepatic microsomal activating system. This effect was first

described by Kitchin *et al.* (1981b) who showed 1.0 mM-NADPH to be toxic in the absence of hepatic microsomes. This effect is first noticeable in the yolk sac, not in the embryonic parameters (Kitchin *et al.* 1981a). Lower concentrations of NADPH are safer for *in vitro* embryonic development but this leads to a decreased efficiency of the metabolic activating system (Kitchin *et al.* 1981a,b). Therefore, Schmid, Goulding, Kitchin & Sanyal (1981) chose to use 0.5 mM-NADPH, a concentration that is both safe for embryo growth and development and sufficient for the functioning of the microsomal mixed-function oxidase system.

Cyclohexylamine in the presence of a metabolic activating system resulted in additive embryotoxicity and dysmorphogenicity. Cyclohexylamine is a substrate for the cytochrome *P*-450 system (Kurebayashi, Tanaka & Yamaha, 1979) while saccharin is not metabolized in the rat and other animal species (Bourgoignie, Hwang, Pennell & Bricker, 1980; Lethco & Wallace, 1975). The microsomal activating system has been shown to potentiate the embryotoxicity of cyclophosphamide (Kitchin *et al.* 1981a) and diallate (Ebron & Kitchin, 1982). The presence of a metabolic activating system in an *in vitro* embryo culture can also inactivate teratogens as has been demonstrated with cytochalasin D (Fantel, Greenaway, Shepard *et al.* 1981).

In our *in vitro* studies 1.0 mM-saccharin, either with or without metabolic activation, caused no biologically significant embryotoxicity or morphological abnormalities (Fig. 1; Table 1). Assuming that the density and volume of distribution of the incubation medium is approximately equal to that of the rat, then a concentration of 1.0 mM-saccharin corresponds to an *in vivo* dose of 182 mg saccharin/kg. Considerable *in vivo* evidence indicates that saccharin does not cause embryonic death or terata in rabbits, mice and rats. Three studies in rabbits showed no adverse effects: Lessel (1971) reported no embryotoxicity in rabbits given 600 mg saccharin/kg during days 1–29 of gestation and found in another study that exposure to 500 or 1000 mg/kg on days 8–16 had no adverse reproductive effect, while Klotzche (1960) administered 5–25 mg/kg on days 8–18 of gestation and did not observe any terata or embryonic deaths. Three studies in mice also showed no adverse reproductive effects: Lorke (1969) found no increase in embryonic death or resorptions with doses of 5–25 mg/kg given on days 6–15 of gestation, Dropkin, Tucci & Porter (1979) found no increases in embryonic death or teratogenicity with much higher doses (500–2000 mg/kg) given on day 10 only, and in a multigeneration feeding study (Kroes *et al.* 1977), 0.2 and 0.5% saccharin in the diet caused no teratogenicity or embryolethality. In addition, four saccharin studies in rats have shown no teratogenic or embryonic effects: Fritz & Hess (1968) found that 5–25 mg/kg given on days 6–15 of gestation produced no embryotoxicity or teratogenicity, Lessel (1971) reported no increase in resorptions or terata with doses of 500–5000 mg/kg given on days 8–16 and found, in a separate study, no resorption of embryos even with a dose of 6000 mg/kg/day given throughout gestation, while Lapointe & Nosal (1979) found that administration of 0.4 mg saccharin/ml drinking-water throughout gestation was not embryotoxic. Hence the findings in the *in vitro* culture system are in agreement with results of *in vivo* experiments for saccharin.

In vitro embryo culture experiments with cyclohexylamine showed few effects at 0.1 and 0.3 mM, but substantial deleterious effects in growth and morphogenesis occurred at a concentration of 1.0 mM both with and without metabolic activation (Fig. 1; Table 1). Using the same assumption as for saccharin, these cyclohexylamine concentrations correspond to *in vivo* doses of 10, 30 and 100 mg cyclohexylamine/kg. *In vivo* teratogenicity studies in Wistar–Imamichi rats given 1.8, 3.6 or 36 mg cyclohexylamine/kg by gavage during days 7–13 of gestation showed no embryonic toxicity or teratogenicity (Tanaka, Nakaura, Kawashima *et al.* 1973). In the same investigation the LD_{50} for pregnant female rats was found to be 180 mg/kg. In a multigeneration feeding study, FDRL rats fed 15, 50, 100 or 150 mg cyclohexylamine/kg body weight/day in the diet did not show any teratogenicity or embryonic death due to these treatments (Oser, Carlson, Cox *et al.* 1976). Hence the *in vitro* findings do not appear to be in agreement with results of *in vivo* studies for cyclohexylamine. However in Swiss SPF mice, cyclohexylamine has exhibited some deleterious effects. Kroes *et al.* (1977) administered 0.5% cyclohexylamine in the diet over several generations and observed some growth retardation and embryo death but not teratogenicity. Becker & Gibson (1970) gave 61, 77 or 122 mg cyclohexylamine/kg ip to Swiss-Webster mice on day 11 and noted an increase in embryo death and resorptions at the two higher doses, but no evidence of teratogenicity was found. In the same study, 61 mg/kg had no adverse effects on any reproductive parameter.

As cyclohexylamine is an organic base, rapid renal excretion of this compound will occur *in vivo* (Renwick & Williams, 1972), a factor which is absent in the *in vitro* culture system. Thus a concentration of 1.0 mM *in vitro* may expose a developing embryo to higher levels of cyclohexylamine for longer periods of time than the calculated corresponding *in vivo* dose of 100 mg/kg, which may explain the observed *in vitro* toxicity. This concentration of cyclohexylamine (1.0 mM) is 56% of the maternal rat LD_{50} (Tanaka *et al.* 1973) which may be an unreasonably high *in vitro* test concentration. Thus we consider the results of the *in vitro* embryo culture studies to be in keeping with the previous *in vivo* results showing that both saccharin and cyclohexylamine are compounds without significant rat embryotoxicity or teratogenicity.

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